Selenastrum Algal Growth Test: Culturing and Test Protocol at the Illinois EPA

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Abstract
The availability of quality test organisms is of fundamental concern in conducting any regularly scheduled biomonitoring activities. The Selenastrum algal assay requires a continuous supply of pure log-phase algae. The most convenient means to meet this demand is through the establishment of in-house cultures. Upon receipt of a pure Selenastrum "starter" culture from an outside source, laboratory stock cultures are initiated. The algae is aseptically transferred to a series of culture flasks containing synthetically prepared algal medium. Once pure algal cultures have been established, a regime of routine cell transfers will provide the laboratory with a steady supply of log-phase algal cells suitable for testing purposes. Back-up reserve cultures are stored on agar slants and plates. Testing of municipal and industrial effluents at the Illinois EPA using Selenastrum algae follows USEPA test protocol. Through experience running the test and repeated attempts to get confident results, the testing has been refined and the integrity of the analysis is ensured. Various techniques are employed in the testing that serve to tighten the USEPA protocol and may be of interest to other regulatory bioassay personnel.

Keywords: Selenastrum algae, log-phase, aseptically, agar slant, agar plate.

Introduction
At the present time, the Illinois EPA (IEPA) is the only state run bioassay laboratory in USEPA Region V conducting the Selenastrum capricornutum algal growth test. The algal growth test is conducted in the Toxicity Testing Unit (TTU) which is one of two units in the Office of Ecotoxicology (OE). OE serves as a support laboratory for the various control divisions within the IEPA (air, land, water, and public water supplies). IEPA uses USEPA protocol, Short Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms (USEPA 1989) as a guideline for culturing and conducting tests. TTU maintains a continuous supply of in-house stock cultures for use in bioassays. Various techniques are employed in culturing and testing that serve to tighten USEPA protocol.

Establishing and Maintaining Selenastrum Stock Cultures
The Selenastrum algal assay calls for healthy log-phase-growth cells which are harvested from resident in-house stock cultures. The TTU laboratory maintains a continuous supply of these log-phase Selenastrum cultures in quantities sufficient to meet all testing demands. The culturing process is relatively straight-forward and consists of six basic components: 1) general culture setup/ conditions; 2) algal nutrient culture medium; 3) aseptic technique; 4) routine cell transfers; 5) back-up/reserve cultures; and, 6) quality assurance/quality control considerations.

1. General Culture Setup/Conditions.
The Selenastrum cultures are maintained in an environmental chamber at 25 ± 1°C under a continuous "cool-white" fluorescent illumination of 400 ± 40 ft-c (4306 ± 431 lux). The algal cells are kept in a constant state of suspension through the use of a mechanical shaker at approximately 100 cpm (cycles per minute). The culture flasks are arranged on the shaker table and allowed to incubate anywhere from four to seven days, depending upon current testing schedules. This incubation interval

Table 1. Nutrient Stock Solutions For Maintaining Algal Stock Cultures (adapted from USEPA 1989).

Nutrient Stock Solution	Compound	Amount dissolved in 500 mL Distilled H_2O
1	$MgCl_2 \cdot 6H_2O$	6.08 g
	$CaCl_2 \cdot 2H_2O$	2.20 g
	H_3BO_3	92.8 mg
	$MnCl_2 \cdot 4H_2O$	208.0 mg
	$ZnCl_2$	1.64 mg[a]
	$FeCl_3 \cdot 6H_2O$	79.9 mg
	$CoCl_2 \cdot 6H_2O$	0.714 mg[b]
	$Na_2MoO_4 \cdot 2H_2O$	3.63 mg[c]
	$CuCl_2 \cdot 2H_2O$	0.006 mg[d]
	$Na_2EDTA \cdot 2H_2O$	150.0 mg
	$NaNO_3$	12.75 g
2	$MgSO_4 \cdot 7H_2O$	7.35 g
3	K_2HPO_4	0.522 g
4	$NaHCO_3$	7.50 g

[a] $ZnCl_2$ - Weigh out 164 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #1.
[b] $CoCl_2 \cdot 6H_2O$ - Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #1.
[c] $Na_2MoO_4 \cdot 2H_2O$ - Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to Stock #1.
[d] $CuCl_2 \cdot 2H_2O$ - Weigh out 60.0 mg and dilute to 1000 mL. Take 1 mL of this solution and dilute to 10 mL. Take 1 mL of the second dilution and add to Stock #1.

provides plenty of viable, log-phase cells suitable for bioassay purposes.

2. Algal Nutrient Culture Medium.
Upon receipt of a Selenastrum "starter" culture from an established outside source, in-house stock cultures are initiated by aseptically transferring a portion of the cells to freshly prepared algal nutrient medium. The culture medium consists of a mixture of various macro- and micronutrients prepared in four separate stock nutrient solutions using the reagent grade chemicals listed in Table 1.

The nutrient medium is prepared by adding 1 mL of each of the four stock solutions, in order as listed in Table 1, per liter of distilled water. The solution is mixed well and then pH-adjusted to 7.5 ± 0.1 by dropwise addition of 0.1 N NaOH or HCL, as appropriate. The medium is then immediately filtered through a pre-washed 0.2 μm pore diameter membrane at a vacuum pressure of approximately 8 psi. The algal nutrient medium is then ready to be dispensed into the various culture flasks and inoculated as needed. Any leftover portions of the sterile medium may be stored in a refrigerator at 4°C until needed. Care should be taken, however, to seal off the storage vessel well so as to prevent loss of water by evaporation. Evaporation losses will alter the concentration of macro-micronutrients in the final medium, thus compromising its quality for use in culturing purposes.

3. Aseptic Technique.
Extreme care is exercised to prevent contamination of the cultures by other microorganisms. All glassware products used in the culturing process are thoroughly cleaned, sealed with aluminum foil, and sterilized at 121°C in an autoclave. All pipet tips used in handling the algal cells during routine cell transfer procedures are of the disposable type, and they too are autoclaved at 121°C. The algal nutrient culture medium is cold-sterilized before use by passing it through a 0.2 μm pore diameter membrane filter, as described above. Despite these efforts, contamination problems do occur from time to time. Contaminated cultures are either discarded or used as food for Ceriodaphnia cultures.

4. Routine Cell Transfers.
To meet scheduled testing demands, the TTU laboratory maintains a continuous source of

log-phase Selenastrum cells. This is achieved through a series of routine cell transfers from existing stock cultures to various aliquots of fresh algal nutrient medium. An inoculum is prepared from a four - to seven - day stock culture by concentrating the cells of the culture through a centrifugation process. The algal cell concentrate is then diluted with distilled water to provide an initial density of approximately 10,000 cells/mL in the culture flasks. A Coulter Counter® (model ZM) is utilized in cell density determinations for both the stock cultures and the final inoculum.

Once prepared, 1 mL of inoculum is aseptically transferred to each of three 500 mL Erlenmeyer culture flasks containing 250 mLs of fresh algal medium each. After inoculation, the flasks are situated in the environmental chamber on a mechanical shaker for incubation purposes. An incubation period of four to seven days renders plenty of healthy, log-phase Selenastrum cells ready for harvest and use in testing and/or other purposes. Routine cell transfers are carried out twice per week, with each transfer staggered 3-4 days apart. This arrangement will provide a continuous supply of log-phase cells suitable for biomonitoring purposes.

The volume of stock cultures required depends on the test loads involved and any other targeted uses for the algae (ie., food source for Ceriodaphnia cultures, etc.). The TTU laboratory meets all of its current algae demands by inoculating 1.5 - 2 L of fresh culture medium weekly.

5. Back-Up/Reserve Cultures.

As mentioned above, contamination of the stock cultures seems to be inevitable from time to time. It is therefore essential to have in place some type of a back-up/reserve system for storing clean, pure Selenastrum stocks that may be called upon to rejuvenate "dirty" or "fouled" cultures. The TTU laboratory meets this objective through the use of a system of agar slants and plates. The agar medium is prepared with the same stock nutrients, in the same amounts, as the standard liquid algal medium. The only difference is that the stock nutrients are dissolved in a 1-2% Bacto®Agar solution. The agar nutrient medium is mixed up in an AgarMatic®bench top agar sterilizer, which in turn is linked up to a PourMatic™automatic plate dispensal system. Thus, the agar medium is mixed, sterilized, and poured into plate form all in one process. Any excess medium is then hand-poured into test tubes for use as slants. A large batch of plates and slants are poured all at once, the bulk of which is then stored in a refrigerator at 4°C until needed.

At scheduled intervals of approximately once a month, several fresh agar plates are "streaked" with Selenastrum cells from existing stock cultures. A 10 μl inoculating loop is used to transfer the cells from the liquid stock cultures to the agar, where they are streaked out into quadrants on the plated medium. The plates are then arranged on a rack situated in a partially enclosed glass box shelter in the environmental chamber for incubation purposes. The glass box, along with rubber bands used to seal the lids on the petri dishes, serves to break up the airflow patterns of the chamber around the immediate vicinity of the plates, thereby minimizing dessication problems of the media. The plated cultures need air exchange for proper growth, but too much airflow will only serve to dry out the plates completely, rendering them useless for storage purposes. An incubation period of 1-2 weeks yields several distinct Selenastrum colonies that may then be targeted for transfer to fresh liquid nutrient medium, thereby rejuvenating active stock cultures.

Agar slants are also streaked up from time to time as needed, but serve primarily in a secondary backup role. After incubation, the slants displaying healthy Selenastrum colonies are pulled from the environmental chamber and stored in a refrigerator at 4°C for up to several months. In this way, they may serve as a "backup" to the backup cultures.

6. Quality Assurance/Quality Control Considerations.

At each cell transfer, the stock cultures are examined microscopically for species verification purposes and to look for any signs of microbial contamination. This information, along with general observations on the overall condition of the cells themselves, is then recorded in an algal culture logbook. This enables the TTU laboratory to keep a running history of culture activities and any special problems/ solutions encountered. Stock cultures are also subjected to monthly NaCl reference toxicant tests. EC50 point estimates are calculated for each reference test, and these values are then plotted on a standard reference toxicant control chart for quality control purposes (Figure 1). These steps are taken to ensure the quality and suitability of the Selenastrum stock cultures for use in biomonitoring activities.

Selenastrum Algal Growth Test Protocol

Samples received by TTU for algal bioassays consist of municipal and industrial effluents and their ambient receiving waters (upstream of the effluent outfall). For the purposes of the IEPA, Illinois is divided into seven regions. All regions, except Region 4 (Field Operations Services in Champaign, IL) and Region 5 (Field Operations Services in Springfield, IL), ship samples to OE via bonded courier (e.g., Emery Worldwide). Regions 4 and 5 hand deliver samples to OE. All samples are received in the laboratory and testing started within 28 hours of sampling. A chain of custody is maintained by field and laboratory personnel to ensure the samples are not tampered with.

When samples arrive in TTU they are logged in, warmed to the proper temperature, aerated, and initial water chemistries are performed. Initial water chemistries consist of alkalinity, hardness, chlorine, and ammonia determinations. Measurement of these parameters helps resolve the cause of toxicity. When the samples have been warmed and aerated, dilutions are poured. A 0.5 dilution series is used. Temperature, pH, conductivity, and dissolved oxygen are measured on each dilution to determine if these parameters are within the range for normal growth of Selenastrum. A 250 mL portion of each dilution is then poured off for the algal bioassay.

Each 250 mL dilution is enriched with 250 μL of each of the four nutrient stock solutions (with EDTA). To reduce the possibility of contamination in the algal bioassay, aseptic techniques are employed throughout the test. All glassware is washed with non-phosphate detergent and rinsed with tap water, acetone, hydrochloric acid, tap water, and distilled water. Glassware and pipet tips are autoclaved at 121°C.

Each dilution (with nutrients) is filtered through a 0.2 μm membrane filter. This filtration removes any indigenous algae from the dilutions. Following filtration, 150 mL of each dilution is measured in each of three 125 mL Erlenmeyer test flasks (three flasks per dilution with 50 mL of diluent per flask). The entire test consists of three test flasks in each of the following concentrations; control, 0%, 6.25%, 12.5%, 25%, 50%, 100%.

The test flasks are inoculated with 1 mL of log-phase-growth Selenastrum (4 to 7 days old) to provide an initial cell density of 10,000 cells/mL (±10%). At IEPA the algal cells are not "washed" prior to inoculation (there is no need to remove EDTA from the test cells since the test nutrients contain EDTA). The required volume of stock culture needed to inoculate the test flasks is calculated as follows:

$$\frac{\text{number of test flasks} \times \text{volume of test solution/flask} \times \text{10,000 cells/mL}}{\text{cell density (cells/mL) in the stock culture}} = \text{volume (mL) of stock culture required}$$

Test flasks are covered with aluminum foil for autoclaving. After the flasks are inoculated, the

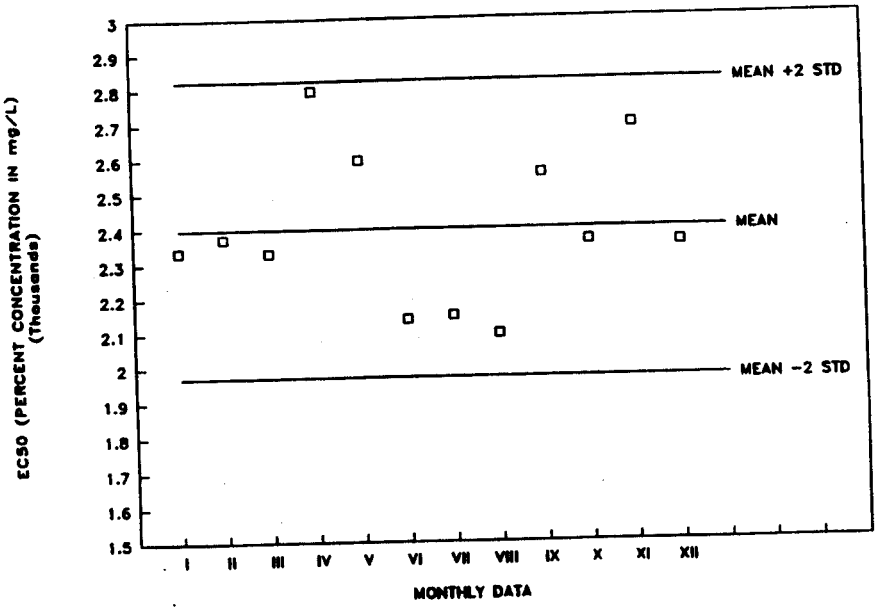

Figure 1. Illinois EPA Reference Toxicity Test (NaCl).

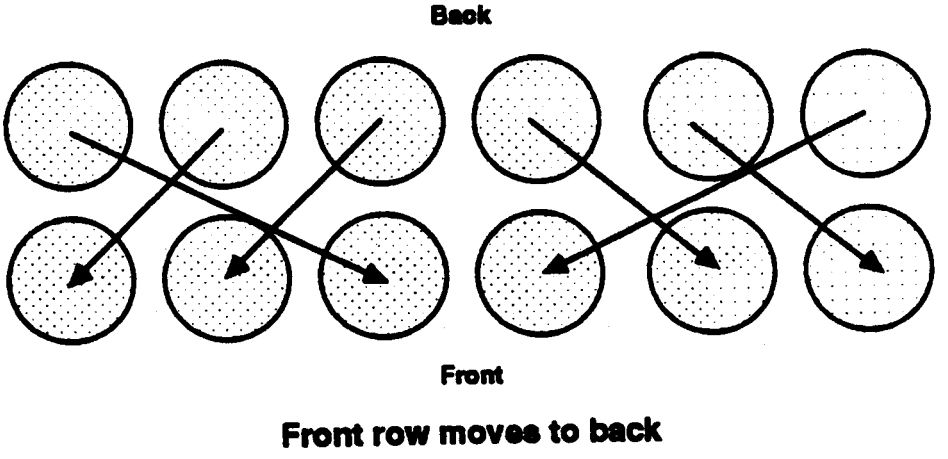

Figure 2. Rotational pattern.

aluminum foil is removed and replaced with 100 mL plastic beakers for incubation. Cell density is checked in at least three flasks within two hours of inoculation. The test flasks are randomized on the holding tray prior to incubation. The flasks are rotated at 24, 48, and 72 hours using a standard rotation pattern (Figure 2) to help ensure that all test containers receive equal amounts and intensity of light and even temperature throughout the 96 hour incubation.

The algal incubator is kept at a constant temperature of 25 ± 1°C. Lighting is turned on approximately four hours before the start of the test to allow the lights to reach equilibrium. During the test the flasks are rotated mechanically at 100 cycles per minute continuous rotation. Light intensity during the test is 400 ± 40 ft-c (4306 ± 431 lux). Light lux is measured at the beginning of the test and at the end of the test. The pH of the 0% and the 100% is also measured at the beginning and at the end of the test.

Test termination is at 96 ± 2 hours. Algal cell density in each flask is measured using a Coulter Counter® Model ZM. Test cultures are diluted with Isoton® (a sodium chloride electrolyte solution) and counted directly on the Coulter Counter®. Three cell counts are taken for each aliquot and the mean cell volume is averaged for the three counts. Each test flask is mixed thoroughly following USEPA procedure. For IEPA purposes, the counts from each test culture must have less than 10% variability.

Test results are considered acceptable if the average cell counts in the control flasks are greater than 2×10^5 cells/mL and control variability does not exceed 20%. When using stock nutrient solutions without EDTA, obtaining average cell counts greater than 200,000 cells/mL was not a problem, however keeping control variability below 20% was difficult. Without EDTA, cell counts in the controls ranged from 400,000 to 800,000 cells/mL. Variability in the three control flasks was as high as 79%. variability in control flasks inoculated with stock nutrient solutions containing EDTA consistently remained below 20%.

EDTA can lower toxicity of a sample by complexing heavy metals. EDTA facilitates algal growth by increasing the availability of micronutrients. Based on the control flask variability (when EDTA is not used) the decision was made at IEPA to conduct the Selenastrum algal bioassay with EDTA in the stock nutrient solutions. Adverse effects on Selenastrum cell growth, expressed in LOEC and NOEC values, are obtained using Dunnett's Procedure. Statistics are analyzed using an in-house written computer program.

Conclusion

The Selenastrum algal bioassay is a useful aquatic toxicity test, and is an important component of IEPA's testing program. In addition to detecting phytotoxic contaminants, the bioassay could identify wastewaters which are nutrient rich and biostimulatory. By incorporating a freshwater primary producer (Selenastrum) into the bioassay regime, toxicity could be detected which is not detected by tests using primary consumers (Ceriodaphnia dubia, at IEPA) or secondary consumers (Pimephales promelas, at IEPA).

Literature Cited

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